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Survey of the Fragile X Syndrome and the Fragile X E Syndrome in a Special Education Needs Population

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To begin to understand the population dynamics of the fragile X (FRAXA) mutation and to learn more about the fragile X E (FRAXE) syndrome, we have initiated a survey of children in special needs education programs in the public school system. With respect to the FRAXA syndrome, we found approximately 1/1,000 full mutations among males. No large alleles at the FRAXE locus were observed among 462 individuals. The allele distributions at the two loci among Caucasians and among African Americans were examined as well as the level of heterozygosity. We found a significant difference in the FRAXA allele distribution among the two ethnic groups; the major difference was due to the lack of smaller alleles among the African Americans. No difference was found for the FRAXE allele distribution among the two groups. The level of heterozygosity was less than predicted by the allele distribution at both loci. This is probably due to unidentified large alleles among females with a test result of a single band. Alternatively, this excess may indicate that the population is not at equilibrium. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X, FRAXA, FRAXE, special education

INTRODUCTION

"The fragile X syndrome is the most common inherited form of mental retardation and, after Down syndrome, the most common identified form of mental retardation." Although statements similar to this are found in almost every introduction to the fragile X (FRAXA) syndrome, little is actually known about the true prevalence of the syndrome or about the frequency of clinically unaffected carriers. To date, almost all estimates of the prevalence of the syndrome are based on cytogenetic surveys of overtly retarded populations. Although the cytogenetic test to identify an individual with the FRAXA syndrome is relatively accurate, it is labor-intensive and its sensitivity and specificity are considerably less than 100%. Once the gene for the FRAXA syndrome was isolated, population surveys of the mutation were anticipated, as the DNA diagnostic test is accurate, relatively simple and inexpensive compared with the cytogenetic test. However, population surveys have been slow to come, in part, due to the technical difficulties related to the type of sample tested (e.g., blood spot) and to ethical and logistical problems related to identification of a target population.

It is now known that in over 95% of cases, the FRAXA syndrome is caused by a single type of mutation—an unstable CGG trinucleotide repeat sequence mutation found in the 5′ untranslated region of the X-linked, fragile X mental retardation gene (FMR1) [Verkerk et al., 1991; Fu et al., 1991]. There are essentially three forms of the repeat region found in the population; they are referred to as "normal," "premutation," and "full mutation." The normal form usually contains about 30 CGG repeats and is interspersed with zero to three single AGG sequences found approximately every ten CGG repeats. The region is polymorphic with respect to the number of repeats (ranging from 6 to 50 repeats) and the number and position of the interspersed AGG sequences [Kunst and Warren, 1994; Fu et al., 1991; Eichler et al., 1994; Snow et al., 1993, 1994; Hirst et al., 1994; Zhong et al., 1995]. Normally, the repeat region is stable when transmitted from parent to child.

It has been suggested that an increase in the number of perfect CGG repeats (either by loss of the AGG interspersion or insertion of additional CGG repeats) causes the repeat region to become unstable and to expand in size when transmitted from parent to child [Kunst and Warren, 1994; Eichler et al., 1994; Snow et al., 1994; Hirst et al., 1994]. This unstable, premutation form of the gene usually consists of 55 to 200 repeats, although alleles with 40 to 60 repeats are some-

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times considered in the grey zone as they may or may not be unstable. Premutation carriers do not exhibit any clinical symptoms related to the syndrome, but are at risk for having affected offspring.

The full mutation form of the FMR1 gene consists of over 200 repeats and is abnormally methylated. Consequently, no mRNA is produced. Lack of the gene product FMRP, an RNA-binding protein [Ashley et al., 1993], is responsible for the mental retardation [Pieretti et al., 1991; Sutcliffe et al., 1992; McConkie-Rosell et al., 1993]. Due to X-linkage, males have more severe expression of the phenotype associated with the syndrome compared with females. Thus, almost all males carrying the full mutation exhibit some type of significant cognitive deficit. The phenotype of females is more variable, presumably due to X-inactivation. Only 50–60% of full mutation carrier females show cognitive impairment, usually mild or borderline mental retardation [Rousseau et al., 1994].

The FRAXE syndrome recently was identified as another trinucleotide repeat sequence mutation, in this case the repeat sequence is CGG [Knight et al., 1993]. Little is known about the frequency of this disorder, but generally is thought to be rarer than the FRAXA syndrome. The phenotype of individuals with this syndrome is described as mild/moderate mental retardation, often with learning disabilities; however, it seems to be extremely variable within and among families [Knight et al., 1993; Flynn et al., 1993].

To begin to understand the population dynamics of the FRAXA mutation and to learn more about the FRAXE syndrome, we have initiated a survey of children in special needs education programs in the public school system.

MATERIAL AND METHODS

In 1993, we initiated a survey of the FRAXA syndrome and, in 1994, of the FRAXE syndrome. Eligible subjects are those children aged 7–10 who are placed in the special education service classes in the public school systems in Metropolitan Atlanta. Children of both genders and all ethnic groups are studied. To date, we have completed the survey in two of the five counties of this geographical-based study. The protocol of the study is defined as follows: 1) a seminar is conducted at each school to describe the purpose of the study, 2) invitation packets and consent forms are given to the teachers of eligible children to be sent to parents, and 3) school visits are scheduled and mouthwash samples obtained from participating children. To ensure confidentiality, no names are provided of eligible children to the study team. Thus, we only had contact with those parents who agreed to participate in the study.

Laboratory Methods

Genomic DNA was extracted from buccal samples collected by either cytology brushes or 0.9% saline mouthwash solution. Each sample was centrifuged at 12,000 to 15,000*g* in 0.9% NaCl. The supernatant was removed and re-suspension buffer (24 mM EDTA, 75 mM NaCl, pH 8.0) was added to the remaining pellet. The sample was then incubated overnight at 37° in 0.1% SDS and 50 μg Proteinase K. DNA was ethanol precipitated and stored in TE^{-4}.

Multiplex PCR reactions for the FRAXA and FRAXE loci were performed on each sample. Primers have been previously described [Fu et al., 1991; Knight et al., 1993]. The 5′ end of the forward primer for each set was fluorescently tagged with a phosphoramidite dye (Applied Biosystems Incorporated, ABI). Amplification of 50–100 ng of genomic DNA was carried out with 0.2 pmol of FRAXA primers and 0.75 pmol of FRAXE primers in a 15 μl solution of 1× Assay Buffer (Fisher Scientific), 10% DMSO, 0.3 mM dATP, 0.3 mM dTTP, 0.3 mM dCTP, 0.3 mM 7-deaza dGTP, and 2.5 units of Taq polymerase (Fisher Scientific). The reactions were heated to 95°C for 7 min followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, elongation at 72°C for 1.5 min. A final elongation step at 72°C proceeded for 7 min. The fluorescent PCR products were denatured in 95% formamide dye. An internal standard for sizing, Rox 1000 (ABI), was included with every sample. The products and standards were loaded onto a 6% polyacrylamide gel. The products were separated on a ABI 373 Stretch automated sequencer. Data were collected using the GENESCAN software package (ABI). Products were sized using the Genotype™ software package (ABI). Male samples that did not amplify and female samples that resulted in only a single band by this method were subjected to a second PCR protocol developed by Brown et al. [1993].

A few samples did not yield a conclusive result by either PCR procedure. These individuals were approached for a blood sample in order to examine the FMR-1 locus by restriction digest. DNA was extracted from blood using the salting-out procedure [Miller et al., 1988]. Approximately 10 μg of DNA was doubly digested with EcoRI (Gibco BRL) and BssHII (Gibco BRL) according to manufacturer's directions [Sutcliffe et al., 1992]. Digested DNA was ethanol precipitated and separated on a 1.2% agarose gel in 1× TBE buffer. Lambda DNA digested with HindIII (Gibco BRL) and radiolabelled with ^{32}P dCTP (Amersham) was used as a sizing standard. DNA was transferred by Hybond N+ (Amersham) membrane by the method of Southern [1975]. The probe pE5.1 [Verkerk et al., 1991] was radiolabelled using the Mega Prime Labelling Kit (Amersham). The membrane was prehybridized and hybridized in Rapid Hyb buffer (Amersham) and washed according to manufacturer's suggestions. DNA was visualized by autoradiography. Typically, exposures were overnight at −70°C.

Statistical Methods

To examine differences in the allele distributions among the two major ethnic groups, we collapsed allele sizes into three categories and conducted a chi-square test of independence. For the FRAXA, the 3 groups were 1) <28 repeats, 2) 28–32 repeats, and 3) >32 repeats. For the FRAXE, the 3 groups were 1) <15 repeats, 2) 15–20 repeats, and 3) >20 repeats.

To compare the level of heterozygosity to that predicted by the allele distribution, we used the methods of

TABLE I. Description of the Study Population

Ethnic group	Male	Female	Percentage
Caucasian	645	295	64
African American	330	142	32
Other	41	18	4
Percentage	69	31	

Nei [1978]. Expected heterozygosity levels were based on male allele frequencies in each population.

RESULTS

Of the 3,333 eligible students, 1,471 agreed to be in the study leading to participation rate of 44%. The description of participants are given in Table I. The greater proportion of males among the participating subjects was representative of that expected for a special needs population. Also, the observed proportion of ethnic groups among the study group was representative of that among the eligible children.

Table II shows the frequency of FRAXA alleles that were greater than 49 repeats and may be at risk for instability. Of the 888 males on whom we have completed testing (888 of 1016, 87%), one had the FRAXA syndrome and three had alleles greater than 50 repeats. Among the 391 females on whom we have completed testing (391 of 455, 88%), one had the FRAXA syndrome and seven had alleles greater than 50 repeats. Among the 312 males and 150 females who were tested for the FRAXE syndrome, no large alleles were identified.

Overall allele distributions for FRAXA (Fig. 1) and for FRAXE (Fig. 4) were similar to those found in other studies [e.g., Fu et al., 1991; Holden et al., 1996]. When we compared the FRAXA allele distribution among Caucasian and African American ethnic groups, we found a significant difference ($\chi^2_2 = 40.5$, $P < 0.001$) (Figs. 2, 3). This difference was due primarily to the lower frequency of smaller alleles in the African American distribution. No difference in the FRAXE allele distribution was found in this small sample ($\chi^2_2 = 5.73$, $P > 0.05$) (Figs. 5, 6).

Next we examined the level of heterozygosity that was expected from the allele distribution and compared it to the observed level of heterozygosity among females (Table III). In all comparisons, the observed heterozygosity was less than that predicted based on the FRAXA and FRAXE distributions for Caucasians and African Americans.

TABLE II. Frequency of Possible FRAXA Mutant Alleles (i.e., Alleles Equal to or Greater Than 50 Repeats)

Allele size	Males (n = 888)	Females (n = 376)
50	0	1
51	0	2
52	1	0
53	0	1
54	0	1
55	1	1
56	0	1
60	1	0
>200	1 (1[a], 1[b])	1 (0[a], 1[b])

[a] Number of previously diagnosed individuals with the FRAXA syndrome who responded, but refused to provide a sample.
[b] Number of previously diagnosed individuals with the FRAXA syndrome who declined participation, but were reported by the teacher to have the FRAXA syndrome.

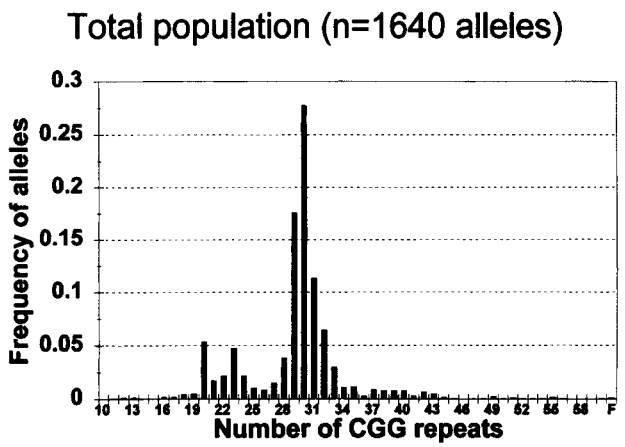

Fig. 1. Distribution of FRAXA alleles in the total study population.

DISCUSSION

We have reported preliminary results from an ongoing population survey of children in special education programs. We found 1/888 and 1/376 special needs males and females, respectively, to have the FRAXA full mutation. Work is still in progress to determine if our participating sample is representative of the eligible population with respect to special needs criteria. Thus, we cannot determine if the estimated frequency is an under- or over-estimate of the true frequency. Recently, Turner et al. [1996] estimated that about 1% of males with special needs had the FRAXA syndrome. The comparatively low frequency found in the preliminary stage of this study may be due to several possibilities: 1) a difference in the level of educational need in the two populations, 2) un-representative sample, 3) small sample size, or 4) a true difference based on the ethnic structure

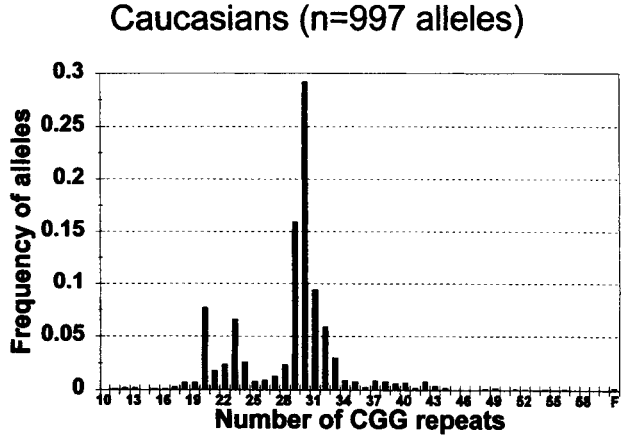

Fig. 2. Distribution of FRAXA alleles in the Caucasian study population.

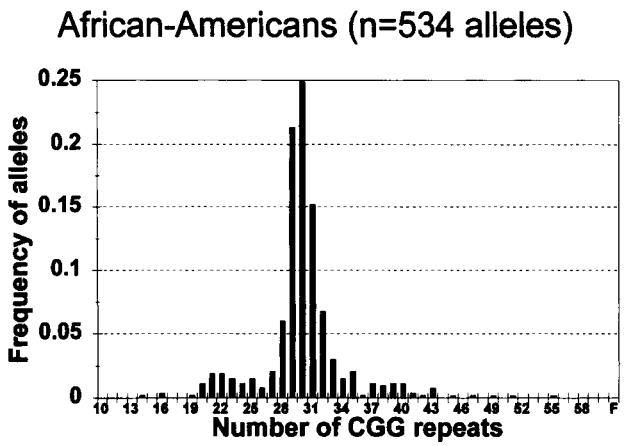

Fig. 3. Distribution of FRAXA alleles in the African American study population.

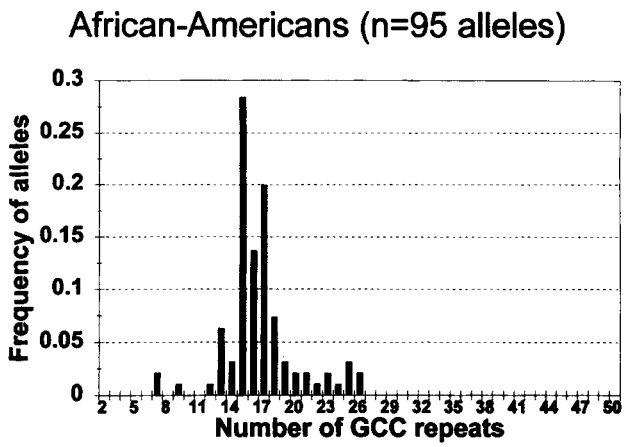

Fig. 6. Distribution of FRAXE alleles in the African American study population.

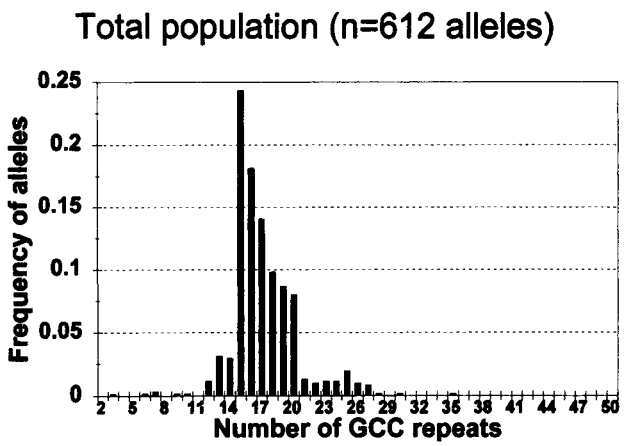

Fig. 4. Distribution of FRAXE alleles in the total study population.

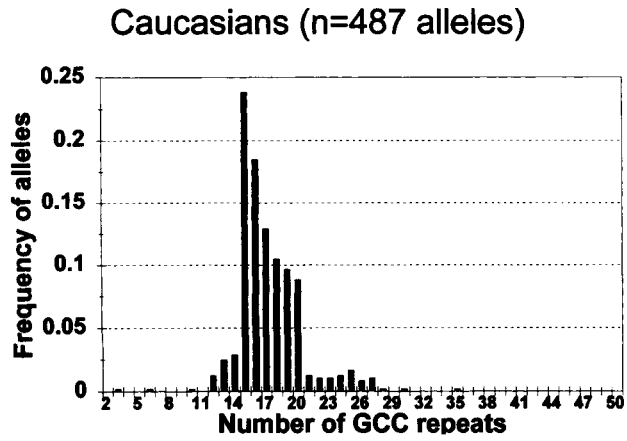

Fig. 5. Distribution of FRAXE alleles in the Caucasian study population.

of the different populations. More work is needed to distinguish these possibilities. No high alleles at the FRAXE were found among the 462 children with special needs. This is not an unexpected finding given the small sample size and lower prevalence of this syndrome compared with the FRAXA syndrome.

FRAXA alleles with 50 or more repeats are candidates for unstable transmissions. Further studies to determine the AGG pattern within the repeat sequence are necessary to identify instability. If we assume conservatively that FRAXA alleles with 55 repeats or more are unstable, the frequency of carriers of such alleles in this population was 2/888 and 2/376 in males and females, respectively. Thus, the allele frequency based on both males and females was estimated to be about 24.4 per 10,000. There were twice as many female carriers compared with male carriers. This is expected because there is little, if any, selection acting against these small mutations. The frequency of these alleles in a special needs population is expected to be similar to that in a random population, as it is assumed that no phenotype is associated with the small mutations. Table IV shows the frequencies of FRAXA alleles with 55 or more repeats estimated from non-impaired populations. Although there is variation in the estimates

TABLE III. Comparison of Observed and Expected Heterozygosity for FRAXA and FRAXE Separated by Ethnic Group

Heterozygosity	Caucasian	African American
FRAXA		
Observed	0.76 ± 0.03	0.73 ± 0.04
Expected	0.86 ± 0.02	0.87 ± 0.02
P-value	0.002	0.001
FRAXE		
Observed	0.63 ± 0.04	0.71 ± 0.09
Expected	0.87 ± 0.02	0.86 ± 0.05
P-value	<0.001	0.258

TABLE IV. Estimated Frequencies of Individuals With 55 to 199 Repeats Obtained From Samples of Non-Mentally Impaired Populations

Reference	Sample	Carrier frequency		Allele frequency (per 10,000)
		Male	Female	
Snow et al., 1993	Blood donors, Caucasian	0/50	1/197	22.5
Arinami et al., 1993	Families with hyperlipidemia, university staff and students, Japanese	0/370	0/227	0 (in 824)
Reiss et al., 1994	Families referred for genetic disorders, mixed ethnic background	0/416	1/561	6.5
Fu et al., 1991	Mixed ethnic background	—	—	0 (in 492)
Rousseau et al., 1995	French Canadian	—	41/10,624	38.6

due to the small sample sizes (with the exception of Rousseau et al. [1995]), the estimate from this sample falls within the limits of those studies.

Comparison of FRAXA allele frequencies among ethnic groups has shown some differences (e.g., Japanese [Arinami et al., 1993], Chinese [Zhong et al., 1994]), although the overall pattern is similar for most groups [e.g., Fu et al., 1991]. We found a significant difference among Caucasians and African Americans and most of the difference can be attributed to the low frequency of small alleles among the African Americans. This is similar to that found for the distribution of alleles in the Chinese population [Zhong et al., 1994]. Such differences in distribution suggest that the frequency of the FRAXA full mutation may also vary among ethnic groups. Although studies in the past have shown the FRAXA syndrome to be present in all ethnic groups, the variation in frequency has not been studied.

Finally, to determine if the population was in equilibrium, we examined the level of heterozygosity of the FRAXA and FRAXE among ethnic groups. We found that the observed heterozygosity was significantly less than the predicted in almost every comparison. This may indicate that the population has not reached equilibrium; however an alternate explanation may also be possible. That is, the detection of large alleles may be low and/or the detection of heterozygotes with only one repeat difference may be difficult. In both of these situations, the female will present with only one band on her test result. Blood from these individuals is currently being requested to ensure that a large allele is not missed.

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